PROJECT NUMBER: 1730
PROJECT TITLE: Plant Cell & Tissue Culture Research
PROJECT LEADER: I. L. Uydess
PERIOD COVERED: October, 1987

I. TOBACCO-IDENTICAL PRESERVATIVES

A. Objective: To develop procedures and to establish microbiological screens for the evaluation of new, tobacco-identical (or "nature-identical") preservatives as replacements for and/or adjuncts to propylparaben.

B. Status:

1. Microbiological Screens: [M. A. Shulleeta, S. W. Tenhet]

   a. Shake-Flask Assay: A synthetic sucrose monoester (obtained from Dr. G. Chan) was evaluated for antimicrobial activity in our standard, Phase I shake-flask preservative screen using B. coagulans as the test organism and 150 µg/ml propylparaben in propylene glycol (PP-PG) as the control.

   b. Agar Diffusion Assay: A standard operating procedure is being developed for an alternative preservative screening assay in which a freshly established lawn of a test micro-organism is challenged with a prospective preservative candidate that has been applied at various concentrations to small (10 mm) filter paper discs. If the test microorganism is sensitive to the compound under evaluation, a clear (cell free) "zone of inhibition" will form around the filter paper disc containing that material (the diameter of the zone generally being related to the relative antimicrobial activity and solubility of that compound).

2. General Microbiology: [J. L. Lyle, L. Weissbecker]

   a. Sporulation: Work continues on the development of a standard operating procedure for the storage of bacteria as spores and/or as freeze-dried vegetative cells.

   b. Microbial Identification: An evaluation of the VITEK microbial identification system is being conducted in collaboration with members of Project 1902.

   c. Isolation of Bacteria from Park 500 SEL: Five pure cultures of Gram Positive bacteria that were isolated from Park 500 SEL have been sent to the American Type Culture Collection (ATCC) for identification.

3. Extraction of Oriental Tobacco: [D. M. Teng]

   An oriental tobacco (LSB) rich in β-methylvaleric acid was used for the isolation and fractionation of surface and cytoplasmic

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lipids for evaluation as potential antimicrobial agents in the paraben replacement program.

4. Preservatives List: [I. L. Uydess]

A list of alternative, preservative candidates has been compiled based upon an evaluation of naturally-occurring compounds (including essential oils and essential oil components) that are known to be approved for use in food-grade and/or tobacco products as a result of their inclusion in one (if not more) of several published approved additives lists (such as the USDA Title-21, UK-Hunter and Fema lists, etc.). A memo describing the rationale of selection and plans for the testing of these compounds has been completed and will be distributed by the end of October.

C. Results:

1. Microbiological Screens:

a. Shake Flask Assay: The synthetic sucrose monoester was tested in duplicate experiments in the Phase I (Nutrient Broth, pH 5.7, 37°C) screen at 50, 250, 500, 1000, 5000, and 10,000 µg/ml. No antimicrobial activity was observed at any of the concentrations evaluated compared to the half-maximal inhibition of growth that was observed in the standard 150 µg/ml PP-PG control.

b. Agar-Diffusion Assay: Several important experimental parameters were evaluated with regard to establishing an S.O.P. for this assay. Three agar formulations (Nutrient Agar, Trypticase Soy Agar, and Oxoid 'D.T.S.' Agar) were compared using various serial dilutions of the overnight (stationary) cultures of four Gram Positive bacteria (B. coagulans, B. licheniformis, B. subtilis, and B. pumilis). Three known antimicrobial compounds (Penicillin, Streptomycin and Chloramphenicol) were employed as the "test" preservatives. Nutrient Agar was generally found to provide the most uniform growth (overall) for the various bacteria examined and yielded the most defined zone of inhibition around the antibiotic discs that were used. A dilution of 1:100 of the overnight cultures of each organism was also found to produce the optimum lawn of bacteria for testing (0.3 ml of this dilution being used to inoculate each 100 mm diameter plate). Furthermore, the preliminary protocol that was generated was successfully tested against B. coagulans using several concentrations of PP-PG applied to sterile filter paper discs.

2. General Microbiology:

a. Sporulation: Four Gram Positive bacilli known to be associated with tobacco products have been sporulated and stored for future use (B. coagulans, B. licheniformis, B. subtilis, and B. brevis).

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b. Microbial Identification: Growth curves have been generated at 37°C and 60°C for six ATCC type strain cultures of bacilli in order to more accurately establish their optimum growth temperature (e.g., as either mesophiles or thermophiles) so that they can be used in a further evaluation of the Vitek microbial identification system. Of the organisms tested thus far, only B. coagulans appears to be a true thermophile based upon the higher growth rate and final (stationary phase) density obtained at 60° vs 37°C.

3. Extraction of Oriental Tobacco:

The surface lipid was removed from the surface of cured samples of oriental (LSB) tobacco using methylene chloride. The resultant extract was subsequently partitioned in hexane and aqueous methanol, and the methanol phase extracted with chloroform. The chloroform extract was then fractionated on a Sephadex LH-20 column. Eight fractions were recovered and stored frozen until they can be tested in future Phase I antimicrobial screens. After the removal of the surface lipids, the leaves were blended in a Waring blender with hexane followed by methanol to extract the cytoplasmic lipids. The combined extract was further extracted with ether and partitioned in hexane and aqueous methanol. Three crude fractions of these cytoplasmic lipids were collected and stored for future testing.

D. **Conclusions**: None to be reported at this time.

E. **Plans**:

1. Additional prospective preservatives will be tested in the Phase I microbiological screen during the remainder of the 4th Quarter, 1987. Target compounds will include sucrose esters and crude extracts isolated from oriental tobacco as well as "nature-identical" compounds that have been approved for use in tobacco and/or food-grade products.

2. A standard operating procedure for the agar diffusion assay will be completed by the middle of November, 1987. This procedure will be employed in the evaluation of prospective preservative compounds as an adjunct to the Phase I (Nutrient Broth) screen that is currently in use.

3. Work will be initiated on the development of a Phase II (SEL) screen for use in future antimicrobial/preservatives tests.

4. The collaborative evaluation of the VITEK microbial identification system will continue until the end of the 4th quarter in support of the objectives of Project 1902.

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